Complexity of apoptotic pathways in leukemia treated with chemotherapy or cellular immunotherapy

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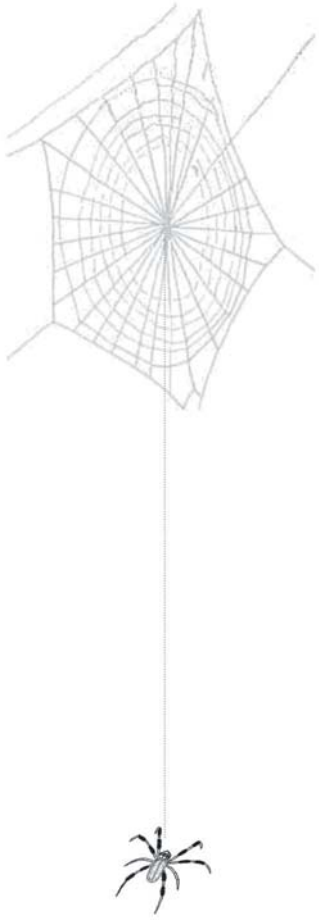
CHAPTER 3



Involvement of caspase-8 in chemotherapy-induced apoptosis of patient derived leukemia cell lines independent of the death receptor pathway and downstream from mitochondria

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ABSTRACT

Resistance of leukemic cells to chemotherapy frequently occurs in patients with acute leukemia, which may be caused by alterations in common apoptotic pathways. Controversy exists whether cytostatic agents induce the mitochondrial or death receptor pathway of apoptosis. In the mitochondrial pathway cytochrome C release and caspase-9 activation play a central role in the induction of apoptosis, while formation of a Death Inducing Signaling Complex (DISC) and caspase-8 activation have been reported to be essential in death receptor-induced apoptosis. Here, we show in human derived myeloid and lymphoblastic leukemia cell lines that caspase-8 plays a more important role than previously expected in apoptosis mediated via the mitochondrial pathway. We demonstrated in these malignant cells chemotherapy-induced apoptosis independent of the death receptor pathway, since blocking this pathway using a retroviral construct encoding Flice inhibitory protein (FLIP) did not inhibit drug-induced apoptosis or caspase-8 activation, while overexpression of Bcl-2 completely inhibited both events. Furthermore, we showed that activation of caspase-8 by cytostatic agents occurred downstream from mitochondria. Since caspase-8 plays a central role in both death receptor- and chemotherapy-induced apoptosis of malignant cells from patients with acute leukemia, therapeutic strategies focusing at modulation and activation of caspase-8 may be successful in the treatment of drug-resistant malignancies.

INTRODUCTION

The treatment of choice for patients with acute leukemia is high-dose chemotherapy followed by allogeneic or autologous stem cell transplantation. [1] From these patients, approximately 70-80% will achieve complete remission. However, 50-60% of all patients experience a relapse of the disease. [2;3] The majority of these patients is resistant to further cytotoxic treatment. Resistance of leukemic cells to chemotherapy may be caused by overexpression of drug efflux pumps like P-glycoprotein or the multidrug-resistance protein MRP-1, [4] or of anti-apoptotic proteins, such as B cell lymphoma-2 (Bcl-2), [5] since overexpression of these proteins has been associated with poor clinical prognosis. [6-8] Functional blocks in caspase activation pathways are found in patients with leukemia and predict poor clinical response to induction chemotherapy, [9] suggesting that alterations of the intrinsic programmed cell death (apoptosis) pathways of the leukemic cells are also a cause of resistance to chemotherapy.

Cytostatic agents initiate the mitochondrial pathway of apoptosis, also called the intrinsic pathway. Due to cellular stress, mitochondria membranes become permeable

and cytochrome c is released. In the cytosol, cytochrome c binds to pro-caspase 9 and apoptotic protease activating factor (Apaf-1). In this complex, called the apoptosome, caspase 9 is activated and in turn cleaves various effector caspases, eventually leading to disassembly of the cell. [10,11]

In addition to the mitochondrial pathway of apoptosis, involvement of the death receptor pathway in chemotherapy-induced apoptosis has been postulated by several investigators, after showing that several anti-cancer drugs induce upregulation of Fas receptor (FasR) and Fas ligand (FasL), followed by subsequent autocrine or paracrine induction of Fas-mediated apoptosis. [12-16] The death receptor pathway is initiated when death ligands, including FasL and tumor necrosis factor alpha (TNF-α), bind to death receptors present at the cell surface of the target cell. Upon extracellular triggering, trimerization of the death receptor occurs, and Fas Associated Death Domain (FADD) is recruited to the intracellular death domain, followed by binding of pro-caspase 8, also called FADD-like IL-1 converting enzyme (FLICE). [17] In this complex of receptor-bound FADD with pro-caspase 8, called the Death Inducing Signaling Complex (DISC), proteolytic cleavage of pro-caspase 8 takes place, initiating a caspase cascade resulting in apoptosis. [18;19]
Recent patient studies showed that absence or low expression of FADD in cells from patients with acute myeloid leukemia is associated with resistance to chemotherapy treatment and poor clinical outcome. [20,21;22] Moreover, downregulation of procaspase-8 expression, by DNA methylation as well as through gene deletion, resulted in several malignancies in resistance to doxorubicin-induced apoptosis. [23-25] Based on these results it may be hypothesized that at least part of the death-receptor signaling pathway is involved in resistance to chemotherapy.

In the present study, we investigated in more detail the role of the death receptor pathway and caspase-8 activation in chemotherapy-induced apoptosis in patient-derived myeloid and lymphoblastic leukemia cell lines. We observed remarkable caspase-8 cleavage and similar amounts of caspase-8 and caspase-9 activity upon apoptosis induction using cytostatic agents indicating that the death-receptor pathway may be involved in chemotherapy-induced apoptosis of these cells. To examine whether this caspase-8 activation was due to activation of the death receptor pathway or could alternatively be involved in the mitochondrial pathway, as previously suggested by Wesselborg *et al*., [26] we introduced the anti-apoptotic proteins FLIP and B-cell lymphoma-2 (Bcl-2) into leukemic cell lines, to specifically inhibit the death receptor or mitochondrial pathway, respectively. FLIP is an enzymatically inactive homologue to caspase 8 and interacts with FADD, preventing pro-caspase 8 to bind to the death domain of the death receptors. [27;28] Bcl-2 was used to specifically inhibit the mitochondrial pathway, since one of the mechanisms of Bcl-2 to inhibit apoptosis, is prevention of cytochrome c release from the mitochondria. [29;30]

Our results show that in these human acute leukemic cells chemotherapy-induced apoptosis is mediated through the mitochondrial pathway and not via the death receptor pathway. The considerable caspase-8 activation upon chemotherapeutic treatment is part of the intrinsic apoptotic pathway and occurs downstream of the mitochondria.

MATERIALS AND METHODS

Cell lines and culture conditions

The human GM-CSF-dependent acute monocytic leukemia cell line AML-193 [31] was obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). The human acute lymphoblastic leukemia cell line Leiden ALL-HP (ALL-HP) and the human CML-derived lymphoblastic cell line Leiden ALL-CM (ALL-CM) were generated in our laboratory from primary human ALL cells. Both cell lines displayed the karyotype of the primary malignant clone, as ALL-HP cells contained i(21)(q10) and ALL-CM cells contained t(9;22)(q34;q11). The cell lines displayed a precursor B immune phenotype and expressed CD10, CD19 and CD79a (both) and CD20 (ALL-CM).

All cell lines were cultured in serum-free medium consisting of IMDM supplemented with 3 mM L-glutamine, 50 μg/mL streptomycin, 50 U/mL penicillin (all Cambrex Bio Science, Verviers, Belgium), 0.4% human serum albumin (HSA) (wt/vol) (CLB, Amsterdam, The Netherlands), 20 μg/mL cholesterol (Sigma-Aldrich, St Louis, MO, USA), 20 μg/mL transferrin (Serva, Heidelberg, Germany), 5 x 10^{-5} M β-mercaptoethanol, and 10 μg/mL insulin (both Sigma-Aldrich). Addition of 20 ng/mL recombinant human GM-CSF (Novartis, Basle, Switzerland) induced persistent proliferation of the AML-193 cells.

Apoptosis inducing agents

Apoptosis was induced with the cytostatic agents camptothecin (Alexis Corp., Lausanne, Switzerland) or daunorubicin (Sigma-Aldrich) using concentrations varying from 0.1 to 100 μM. Death-receptor-mediated apoptosis was induced using Fas agonistic antibodies (10 to 1000 ng/mL) that cause crosslinking of the Fas receptor (Fas Ab, 7C11; Beckman Coulter Inc., Fullerton, CA, USA), or by TRAIL (rhs*Killer*TRAIL™) (Alexis) using concentrations varying from 1 to 1000 ng/mL.

Cytotoxicity assays

Cytotoxicity was measured using 16 or 24-hr ^{51}Cr release assays as described previously [32] or using CFSE-based cytotoxicity assays as described by Jedema *et al*., [33] with some modifications. Target cells were labeled with 5 μM CFSE (Molecular Probes Europe, Leiden, The Netherlands), and incubated overnight in a humidified atmosphere of 5% CO_2 and 37°C. For the cytotoxicity assay, 25000 cells/well (100 μL) were plated in 96-well microtiter plates (all in triplicate), and apoptosis-inducing agents were added in a volume of 50 μL/well. After 16 or 24 hrs of exposure, FACS analysis was performed to determine numbers of viable cells. To exclude dead cells from the analysis, Propidium Iodide (PI) (1 μg/mL; Sigma) was added. To allow quantitative analysis of the viable cells, the wells were harvested, and transferred to FACS tubes containing 10000 Flow-Count Fluorospheres (Coulter Corporation, Miami, FL, USA). For each sample 3000 microbeads were acquired, facilitating the calculation of absolute numbers of viable (PI^-) $CFSE^+$ target cells. The percentage of specific cell death was defined as:

[(mean absolute number of viable $CFSE^+$ target cells in control medium - absolute number of viable $CFSE^+$ target cells ^{experimental}) / (mean absolute number of viable $CFSE^+$ target cells in control medium)] x 100.

To determine the role of caspases in chemotherapy- or death receptor-induced apoptosis, ^{51}Cr-labeled target cells were pre-incubated for 2 hrs with 100 μM of irreversible cell-permeable broad-spectrum caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK), caspase-3-inhibitor (z-DEVD-FMK), caspase-8 inhibitor (z-LETD-FMK) or caspase-9 inhibitor (z-LEHD-FMK) (all Alexis) prior to apoptosis induction.

Annexin V / PI staining
Apoptosis was determined by Annexin V and PI staining. Annexin V specifically binds to phosphatidyl serine (PS), a phospholipid that becomes exposed on the surface of cells undergoing apoptosis. Dual staining with PI enables the identification of early apoptotic cells that have not yet lost their membrane integrity. [34] Cells were collected and resuspended in binding buffer (10 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) at a concentration of 10^6 cells/mL. Cells were incubated at room temperature for 20 min with 0.1 μg/mL FITC-labeled Annexin V (Bender MedSystems, Vienna, Austria), washed, and resuspended in binding buffer. PI was added at a final concentration of 1 μg/mL, and the cells were directly analyzed on a flowcytometer.

SDS PAGE and Western Blot analysis
Apoptosis was induced using camptothecin (50 μM) or Fas Ab (500 ng/mL for AML-193 or 100 ng/mL for ALL-HP cells) in a time range from 0 to 24 hrs of exposure. At each time point, whole cell lysates of 2 x 10^6 cells were obtained by freeze-thawing the cells in 100 μL NP40-lysisbuffer (50 mM Tris-HCl, pH 7.6, 5 mM dithiotreitol, 20% v/v glycerol, 0.5% v/v Nonidet P40, and 25% v/v Protease Inhibitor Cocktail (Boehringer, Mannheim, Germany). SDS PAGE and Western Blot analysis using PVDF membranes (Millipore Corp., Bedford, MA, USA) were performed as previously described. [35] Primary antibody incubations were performed for 2 hrs in 1% Ecl-blocking reagent. Caspase cleavage was detected using antibodies specific for caspase-8 (1:2000; BD BioSciences Pharmingen, San Diego, CA, USA), caspase-9 (1:8000; Calbiochem, San Diego, CA, USA), or cleaved caspase-3 (1:1000; Cell Signaling Technology, Inc., Beverly, MA, USA). Antibodies specific for the FLAG epitope tag, used to detect introduced FLIP (1:1000), were purchased from Sigma.
After 3 washing steps, membranes were incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:3000; Promega, Madison, USA). β-actin expression was determined on the same blots after stripping for 30 minutes at 65°C with buffer containing 0,5% SDS using anti-β-actin clone AC-15 moAbs (1:100 000; Sigma-Aldrich).

Caspase activity assay using colorimetric substrates
After apoptosis induction, for each time point 4 x 10^6 cells were washed and resuspended in 100 μL of ice-cold lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT (freshly added), 0.1 mM EDTA). After 5 min of incubation on ice, cells were centrifuged at 10,000 x *g* for 10 min at 4°C, and supernatants (= cell extracts) were either directly used for the assay or quickly frozen in liquid nitrogen, and stored at -80°C, for later use. Caspase activity assays included 50 μL of assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT (freshly added), 0.1 mM EDTA and 10% glycerol), 40 μL of cell lysate (~ 60-70 μg of protein) and 10 μL of substrate (200 μM final concentration of DEVD-pNA, IETD-pNA or LEHD-pNA, all purchased from Alexis). Samples were incubated at 37°C, and enzyme-catalyzed release of p-nitroanilide was monitored for several hrs at 405 nm using a microtiter plate reader. Absorbance at 405 nm was plotted versus time for each sample. The slope (OD/time) was calculated for the initial time period over which the plot was linear, and this slope was used as a measure for caspase activity.

Flow cytometric analysis of intracellular Bcl-2
To determine Bcl-2 expression, cells were permeabilized at room temperature for 5 minutes using 0.25% saponine/PBS. After centrifugation at 1400 rpm, cells were stained for 30 min at 4°C with FITC-conjugated anti-Bcl-2 (Dako, Glostrup, Denmark) or IgG1-FITC isotype control (BD) MoAbs. After washing with PBS containing 0,8 g/L human albumin, cells were subjected to flow cytometric analysis using a BD FACScan.

Construction of retroviral vectors and generation of retroviral supernatants
The complete coding region of human FLIP-long (U97074) with a FLAG tag in front of the start codon, was amplified from plasmid pCR3.V64 (kindly provided by Dr. J.P. Medema (Leiden University Medical Center, Leiden, Netherlands) already containing the long form of FLIP with a FLAG sequence at the N-terminus, by PCR using the forward primer 5'-tatagaagatctaccatggattacaaagacgatgac-3' and reverse primer 5'-ta taccgctcgagttatgtgtaggagag-3'. PCR products were sequenced to exclude mutations and were cloned

into the Moloney murine leukemia virus-based retrovirus vector LZRS (G. Nolan, Stanford University, Palo Alto, CA) containing truncated nerve growth factor receptor (ΔNGF-R) as the marker gene. [36] The Bcl-2 construct was kindly provided by Dr. J.P. Medema, and cloned into the pLZRS vector with green fluorescence protein (eGFP) as the marker gene. Retroviral pLZRS vectors encoding eGFP or ΔNGF-R alone were used as control vectors (mock) in the experiments. The pLZRS constructs were transfected into packaging cells ϕ-NX-A (G. Nolan, Stanford University, Palo Alto, CA) [36] using calcium phosphate (Life Technologies, Gaithersburg, MD) and retroviral supernatants were obtained as previously described. [37]

Retroviral transduction of leukemic cells with anti-apoptotic genes
Exponentially growing AML-193, ALL-HP and ALL-CM cells were transduced with retroviral supernatants based on the method described by Hanenberg *et al*. [38] with minor modifications, [37] using recombinant human fibronectin fragments CH-296 (RetroNectin; Takara, Otzu, Japan). ΔNGF-R expression was detected using a phycoerythrin (PE)-conjugated anti-human NGF-R mAb (Pharmingen). Transduced cells were purified by FACS® sort based on marker gene expression using a FACSVantage (Becton Dickinson, Mountain View, CA), and cultured in serum free medium.

RESULTS

Sensitivity of various leukemic cell lines to various apoptosis inducing agents

To determine sensitivity of various leukemic cell lines to various apoptotic stimuli, both ^{51}Cr release assays (AML-193 and ALL-HP) and CFSE assays (ALL-CM) were performed. In Figure 3.1 representative percentages of cell lysis after 24 hrs of exposure to various concentrations of camptothecin (A), daunorubicin (B), Fas agonistic antibody (Fas Ab) (C) or TRAIL (D), are depicted for each cell line. In AML-193 (white bars) and ALL-HP cells (grey bars), the cytostatic agents camptothecin and daunorubicin were equally effective in inducing apoptosis, while ALL-HP cells were slightly more sensitive to the death receptor triggering agents Fas Ab and TRAIL. ALL-CM cells (black bars) were effectively killed by chemotherapeutic agents and TRAIL, but not by Fas Ab due to absent Fas-receptor expression (data not shown). Concentrations of apoptotic agents resulting in at least 40% lysis after 24 hrs of exposure, indicated with an asterisk (*), were used in further experiments.

To investigate whether these leukemic cells died via an apoptotic mechanism in response to these different agents, we performed annexin V/PI stainings after 4, 6, 16 and 24 hrs of exposure (partially shown in Figure 3.2). We counterstained the cells with Propidium Iodide (PI) to distinguish between early apoptotic and dead cells. These data demonstrate that chemotherapy-induced cell death was mediated via apoptosis in all cell lines studied, since cells became annexin V positive after 4 hrs of treatment, while at these time points no cell death was observed (PI negative and no ^{51}Cr release). Triggering of death receptors by either Fas Ab (AML-193 and ALL-HP) or TRAIL (ALL-CM) also resulted in the induction of apoptosis. The kinetics of Fas/TRAIL-induced apoptosis were slower than of chemotherapy-induced apoptosis, especially for ALL-HP cells, which became annexin V positive after 16 to

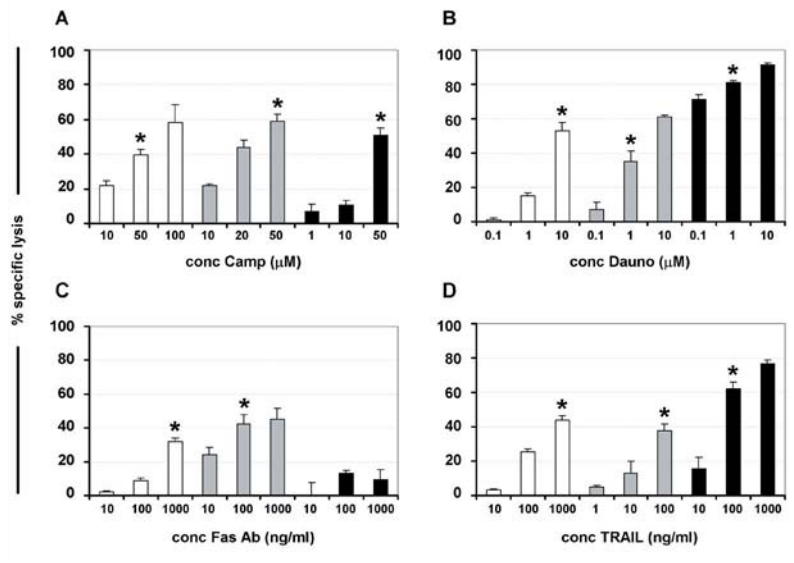

Figure 3.1. Sensitivity of various leukemic cell lines to various apoptotic agents.
AML-193 (white bars), ALL-HP (grey bars) and ALL-CM cells (black bars) were exposed for 24 hrs to various concentrations of (A) camptothecin (Camp), (B) Daunorubicin (Dauno), (C) Fas agonistic antibody (Fas Ab) or (D) TRAIL. Percentages of specific lysis were determined using both ^{51}Cr release assays (AML-193 and ALL-HP) and CFSE assays (ALL-CM). The data shown are representative of three independent experiments. Concentrations of apoptotic agents resulting in at least 40% lysis, marked with an asterisk (*), were used in further experiments.

24 hrs of exposure to Fas Ab. Percentages of cell death after 24 hrs of incubation were comparable between the quantitative annexinV/PI analyses (using Flow-Count Fluorospheres; data not shown) and the ^{51}Cr release assays (Figure 3.1).

Caspase-8 and caspase-9 are involved in chemotherapy-induced apoptosis of various leukemic cell lines

To investigate the contribution of the various apoptotic pathways in chemotherapy-induced apoptosis, we first assessed caspases -8, -9 and -3 activation patterns upon apoptosis induction using camptothecin. Since other cytostatic agents such as daunorubicin caused similar caspase-activation patterns as campthothecin, these results were not shown. Figure 3.3A shows that in all cell lines rapid and substantial caspase-8, -9 and -3 cleavage was detected after treatment with camptothecin (left panel). Caspase-activation was maximal after 4 or 6 hrs of exposure to camptothecin in all cell lines. In AML-193 cells, the first cleavage products of caspase-8 and caspase-9 were present after 2 hrs of exposure, while caspase-3 activation was observed after 4 hrs of treatment. In ALL-HP cells, first cleavage products of caspase-8, -9 and -3 were observed simultaneously after 2-4 hrs of exposure. Kinetics of caspase-activation was the fastest in ALL-CM cells, showing

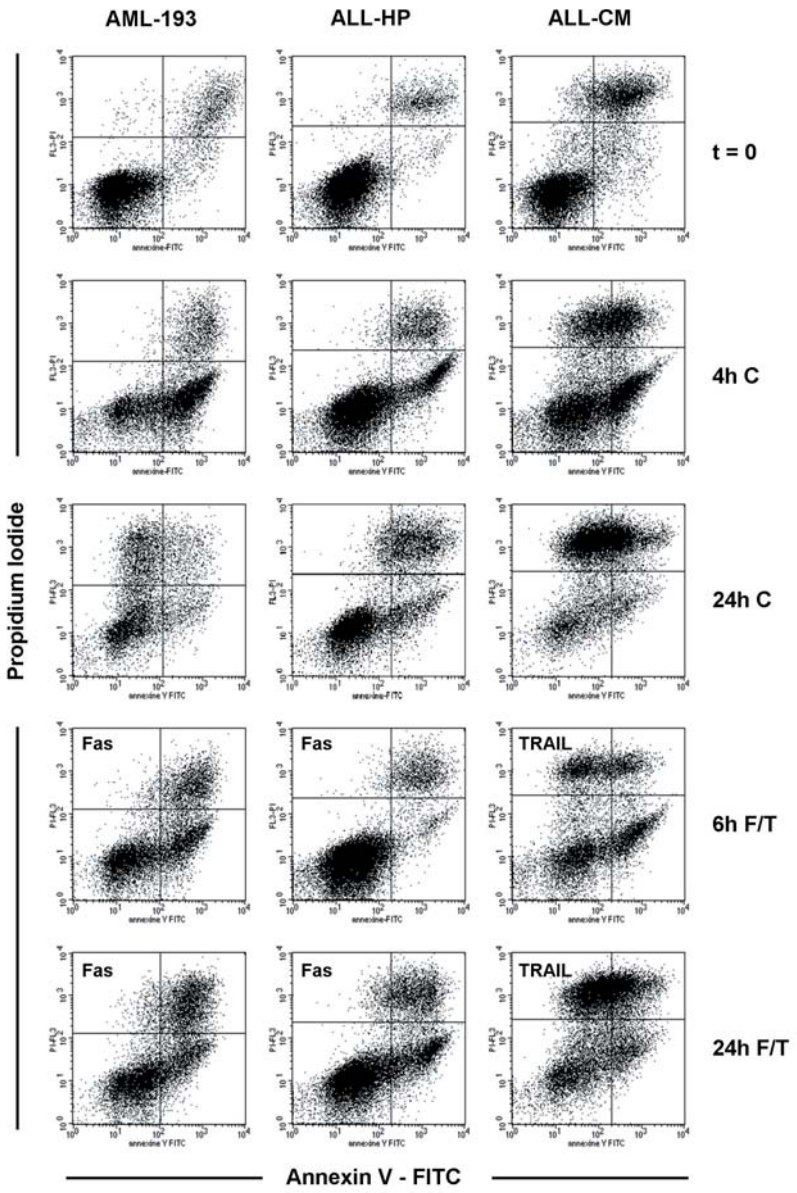

Figure 3.2. Leukemic cells die via an apoptotic mechanism in response to cytostatic agents and Fas Ab or TRAIL.

AML-193, ALL-HP and ALL-CM cells were exposed for 4 or 24 hrs to camptothecin (C), and for 6 or 24 hrs to Fas Ab (F) or TRAIL (T) in case of ALL-CM. Phosphatidyl serine exposure was determined by FACS analysis using annexin V-FITC staining combined with PI. Dotplots are representative of at least 2 independent experiments.

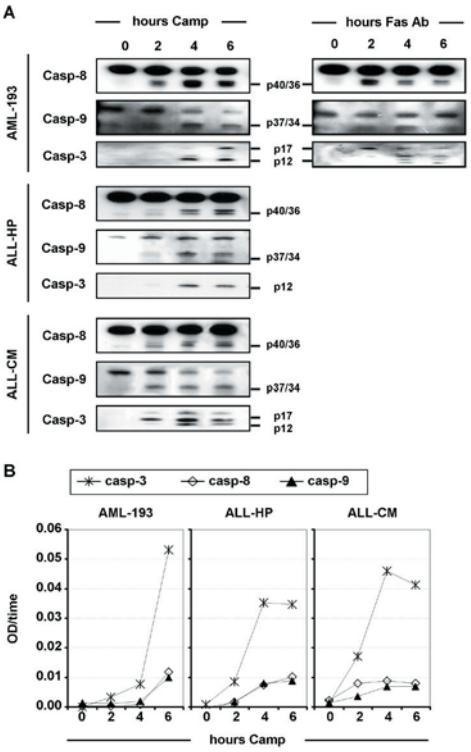

Figure 3.3. Caspase-activation patterns upon chemotherapy or death receptor-induced apoptosis in various leukemic cell lines.
(A) Caspase cleavage patterns for AML-193, ALL-HP and ALL-CM cells exposed to 50 μM of camptothecin (Camp, left panel) for indicated periods of time, and for AML-193 cells treated with 500 ng/mL of Fas Ab (right panel). Cell lysates (15 μg total protein per lane) were separated by 12% SDS-PAGE. Western Blot analysis for caspase-8, -9 and -3 was performed as described in Materials and Methods. The migration position of full-length caspase-8 (Casp-8), its cleavage intermediate p40/36, full-length caspase-9 (Casp-9), its cleavage intermediate p37/34, and the active subunits p17 and p12 of caspase-3 (Casp-3) are indicated. (B) Caspase activity assays in AML-193, ALL-HP and ALL-CM cells treated with 50 μM of camptothecin (Camp) for indicated periods of time. Cell extracts were prepared as described under Materials and Methods and assayed for caspase activity (in duplicate) using the colorimetric substrates DEVD-pNA (caspase-3-like protease), IETD-pNA (caspase-8-like protease), and LEHD-pNA (caspase-9-like protease). Data shown are representative of 2 independent experiments.

cleavage of all three caspases already after 2 hrs of treatment with camptothecin. As illustrated in AML-193 cells, the kinetics of caspase-8 activation upon triggering with camptothecin (left panel) was comparable to Fas Ab-induced caspase-8 activation (right panel), although maximal caspase-8 activation was already observed after 2 hrs of exposure to Fas Ab.
To study the kinetics of caspase-8, caspase-9 and caspase-3 activation in more detail upon exposure to cytostatic agents, we performed caspase-activity assays using

colorimetric caspase substrates (Figure 3.3B). These assays showed similar kinetics of cleavage of caspase-8 and caspase-9 substrates in all cell lines suggesting that both caspases are activated in the same time interval after apoptosis induction with camptothecin. In none of the cell lines cleavage of caspase-8 or caspase-9 substrates occurred earlier than cleavage of caspase-3 substrates (Figure 3.3B), suggesting that all three caspases were simultaneously activated. Conversion of caspase-8 and caspase-9 substrates was much lower than caspase-3 activity in all three cell lines.

To determine whether both caspase-8 and caspase-9 play an important role in the execution pathways induced by chemotherapeutic agents the effect of specific caspase-inhibitors on the percentages of camptothecin and Fas Ab- or TRAIL-induced apoptosis was studied. Percentages of specific inhibition, defined as the percentage of decrease in apoptosis induction in presence of specific caspase-inhibitors, were corrected for inhibition of spontaneous apoptosis mainly observed using the broad-spectrum caspase-inhibitor zVAD-FMK (zVAD). Percentages of specific inhibition of apoptosis by zVAD, and by caspase-3, -8 and -9 inhibitor after 24 hrs of exposure to various apoptotic agents are shown in Figure 3.4. In general, apoptosis was most effectively inhibited by zVAD (black bars). As expected, caspase-8 inhibitor caused 70-80% inhibition of death receptor-induced apoptosis by Fas Ab and TRAIL in all cell lines. Inhibition of death-receptor-induced apoptosis by caspase-9 inhibitor ranged from 20-50%.

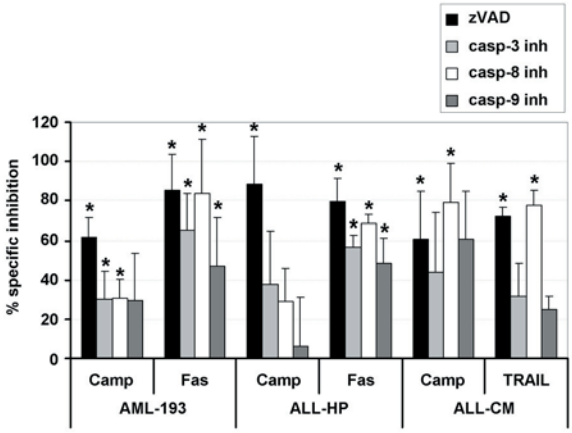

Figure 3.4. Effect of various caspase-inhibitors on chemotherapy or death receptor-induced apoptosis in various leukemic cell lines.
AML-193, ALL-HP and ALL-CM cells were exposed for 24 hrs to 50 μM camptothecin (Camp) or Fas agonistic antibody (Fas) (1000 ng/mL for AML-193 and 100 ng/mL for ALL-HP) in presence or absence of specific caspase-inhibitors (100 μM). Since ALL-CM cells were insensitive to Fas (Figure 3.1), TRAIL (100 ng/mL) was used to activate the death receptor pathway. Percentages of inhibition of lysis by the broad-spectrum caspase-inhibitor zVAD-FMK (zVAD), and by caspase-3, -8, or -9 inhibitors were calculated. Results are the mean percentage of inhibition by each caspase-inhibitor + SD of 3 independent experiments; *, $p<0.05$ compared to control cells without inhibitor.

Inhibition of camptothecin-induced apoptosis by caspase-8, caspase-9 and caspase-3 inhibitors varied between the cell lines studied. In AML-193 and ALL-HP cells minimal inhibition ranging from 10 to 40% was observed, whereas in ALL-CM cells inhibition varied from 40 to 80%. Within each cell line only marginal differences in inhibition with caspase-8, caspase-9 and caspase-3 inhibitors were found. However, in none of the cell lines inhibition with caspase-9 inhibitor was significant, whereas in AML-193 and in ALL-CM caspase-8 inhibitor significantly inhibited camptothecin-induced apoptosis. These data and the simultaneous activation of both caspase-8 and caspase-9 upon exposure to camptothecin suggested that both the death receptor and the mitochondrial pathway play a role in chemotherapy-induced apoptosis of these cell lines.

Chemotherapy-induced apoptosis is not linked to the death receptor pathway but completely dependent on the mitochondrial pathway in leukemic cell lines

To investigate whether the concurrent activation of caspase-8 and caspase-9 indicated that both pathways play a role in chemotherapy-induced apoptosis, we introduced anti-apoptotic constructs into our panel of leukemic cell lines. FLIP expression was used to block the death receptor pathway, whereas the Bcl-2 encoding construct was introduced to specifically inhibit the mitochondrial pathway. Transduced cells were FACS sorted on the basis of ΔNGFR or GFP expression, which resulted in >90% pure populations. Protein expression of the transduced cell lines was determined to verify proper translation of the introduced anti-apoptotic constructs, which is depicted in Figure 3.5A for the various transduced ALL-CM cells.

To investigate whether these anti-apoptotic constructs could prevent early-apoptotic processes, we analyzed annexin V expression in the different cell lines upon apoptosis induction with various apoptotic agents. An example is given in Figure 3.5B, in which ALL-HP cells transduced with mock, Bcl-2 or FLIP were exposed to camptothecin or Fas agonistic antibody. Increased Bcl-2 expression largely prevented phosphatidyl serine exposure on the cell surface after apoptosis induction with camptothecin, but not after treatment with Fas Ab. In contrast, enhanced FLIP expression resulted in specific inhibition of Fas Ab-induced apoptosis, while chemotherapy-induced apoptosis could not be prevented. An overview of the inhibitory effect of both elevated Bcl-2 and FLIP expression on the amount of chemotherapy- and death receptor-induced apoptosis in the three different cell lines is given in Figure 3.5C. All FLIP-transduced cell lines studied showed around 70% inhibition of lysis induced with Fas Ab or TRAIL, illustrating effective inhibition of the death receptor pathway by FLIP. In contrast, FLIP-transduced cells and mock-transduced cells were equally sensitive to camptothecin and daunorubicin also using other concentrations than shown in the figure, demonstrating that the death receptor pathway is not involved in chemotherapy-induced apoptosis in AML-193, ALL-HP and ALL-CM cells. To investigate the involvement of the mitochondrial pathway in chemotherapy-induced

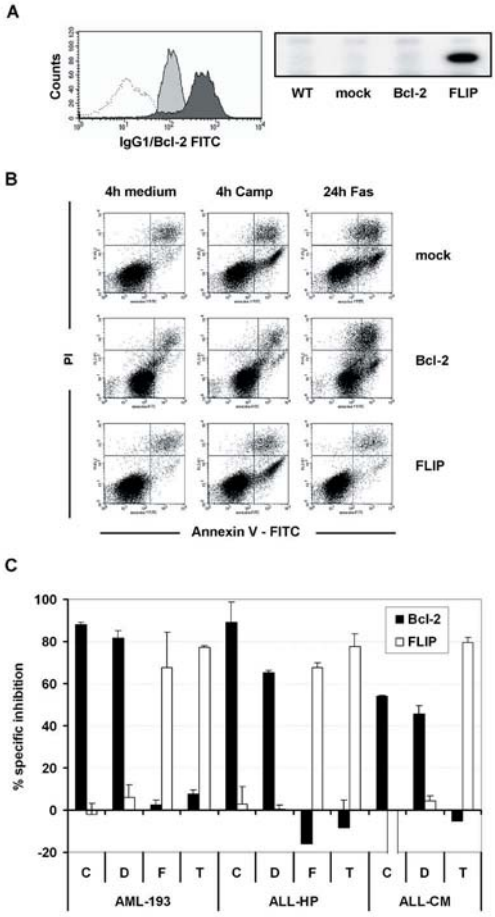

Figure 3.5. Selective inhibition of chemotherapy-induced apoptosis by Bcl-2 and of death receptor-induced apoptosis by FLIP in various cell lines.
(A). Bcl-2 expression (left) of mock (light grey) and Bcl-2 (dark grey) transduced ALL-CM cells was determined using flow cytometry. As a control, IgG1-FITC staining is shown (dotted line). FLAG-tagged FLIP expression (right) of WT, mock-, Bcl-2-, or FLIP-transduced ALL-CM cells is shown, as determined by Western Blot analysis using FLAG-tag specific antibodies. (B) The effect of elevated Bcl-2 and FLIP expression on apoptosis induction after treatment for 4 hrs with camptothecin (Camp) or for 24 hrs with Fas Ab is shown for ALL-HP cells, as determined by FACS analysis using annexin V-FITC staining combined with PI. Note the elevated FL-1 intensity of untreated (4 hrs medium) ALL-HP-Bcl-2 cells caused by GFP marker gene expression of the Bcl-2 construct. (C) The effect of increased Bcl-2 and FLIP expression on total cell death of various leukemic cells after exposure to various apoptotic agents. AML-193, ALL-HP and ALL-CM cells transduced with vector control (mock), Bcl-2, or FLIP were exposed for 24 hrs to camptothecin (Camp), Daunorubicin (Dauno), Fas agonistic antibody (Fas) or TRAIL using concentrations as indicated in Figure 3.1 (*). Because of insensitivity, ALL-CM cells were not treated with Fas. Cytotoxicity was determined using both ^{51}Cr release assays (AML-193 and ALL-HP) and CFSE assays (ALL-CM). Percentages of inhibition by increased expression of Bcl-2 (black bars) or FLIP (white bars) on Camp-, Dauno-, Fas- or TRAIL- induced apoptosis were calculated, and a representative result of three independent experiments is shown in the figure.

apoptosis in these cell lines, the inhibitory effect of Bcl-2 expression was determined. As illustrated in Figure 3.5C, Bcl-2 almost completely inhibited apoptosis induced by 16 to 24 hrs exposure to various cytostatic agents in AML-193 and ALL-HP cells, and caused 50% inhibition in ALL-CM cells, emphasizing an important role of the mitochondria in the execution of chemotherapy-induced apoptosis. The same results were found using other concentrations of cytostatic agents (data not shown).
In conclusion, despite substantial activation of caspase-8 in all 3 leukemic cell lines studied, chemotherapy-induced apoptosis was not executed via the death receptor pathway but was completely dependent on the mitochondrial pathway.

Chemotherapy-induced caspase-8 activation occurs downstream from the mitochondria in all leukemic cell lines studied

To verify that chemotherapy-induced caspase-8 activation was not death-receptor-mediated, we tested the FLIP-transduced cell lines for their capacity to inhibit camptothecin-induced caspase-8 cleavage. Figure 3.6A shows that caspase-8 activation upon camptothecin induction was not affected by increased expression of FLIP in AML-193 and ALL-HP cells, despite decreased basal expression of procaspase-8 in FLIP-transduced AML-193 cells compared to mock- or Bcl-2-transduced cells. Expression of β-actin was used to confirm equal protein loading. As a control, FLIP-transduced AML-193 and ALL-HP cells showed a complete block in Fas-induced caspase-8 activation compared to the mock-transduced cells (Figure 3.6B). These data indicate that caspase-8 activation upon camptothecin-triggering likely occurs in the cytosol, independent of death receptors and DISC formation.
To reveal whether caspase-8 cleavage occurred upstream or downstream from the mitochondria, the effect of Bcl-2 expression on camptothecin-induced caspase-8 activation was assessed (Figure 3.6A). Caspase-8 cleavage was almost completely blocked in all Bcl-2 expressing cell lines, indicating that caspase-8 was mainly activated downstream from the mitochondria upon chemotherapy-induced apoptosis. In contrast, caspase-8 cleavage induced by Fas Ab was mainly occurring independent of mitochondrial activation (Figure 3.6B), since Fas-induced caspase-8 activation in Bcl-2-transduced cells was almost similar as in mock-transduced cells.
To confirm that Bcl-2 completely inhibited caspase-8 activation after treatment with cytostatic agents, we performed in addition caspase-8 activity assays (Figure 3.6C). Although conversion of IETD-pNA substrate was low in both cell extracts of AML-193 and ALL-HP cells treated with camptothecin, clear inhibition of caspase-8 activation was observed in the Bcl-2- compared to the mock- and FLIP-tranduced cell lines.

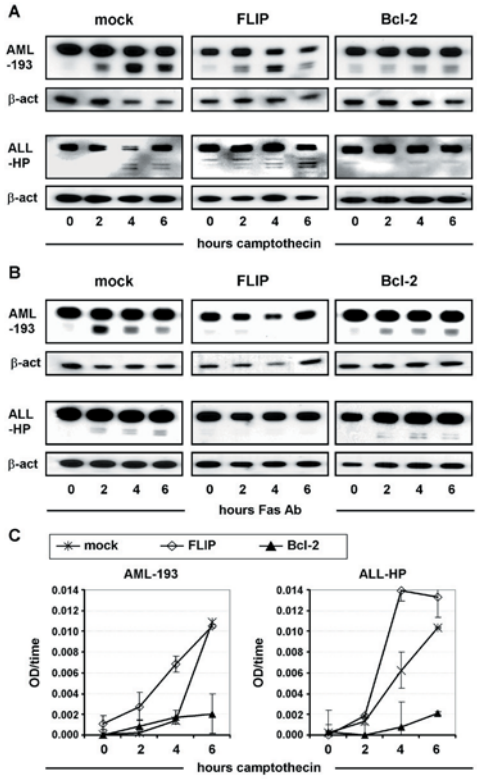

Figure 3.6. Selective inhibition of chemotherapy-induced caspase-8 activation by Bcl-2 and of death receptor-induced caspase-8 activation by FLIP expression.
AML-193 and ALL-HP cells transduced with vector control (mock), Bcl-2, or FLIP were exposed for indicated periods of time to (A) camptothecin (50 μM) or (B) Fas Ab (500 ng/mL). Cell lysates (15 μg total protein per lane) were separated by 12% SDS-PAGE. Western Blot analysis for caspase-8 was performed as described in Materials and Methods. Full-length caspase-8 and its cleavage intermediate p40/36 are shown in the figure (see Figure 3.3A). Equal protein loading was confirmed by expression of β-actin. Note that increased expression of FLIP appeared to decrease procaspase-8 expression in AML-193 but not in ALL-HP cells, compared to mock or Bcl-2 expressing cells. (C) Caspase-8 activity assay in AML-193 and ALL-HP cells transduced with vector control (mock), Bcl-2, or FLIP, and treated with 50 μM of camptothecin (Camp) for indicated periods of time. Cell extracts were prepared as described under Materials and Methods and assayed for caspase-8 activity (in duplicate) using the colorimetric substrate IETD-pNA. Data shown are mean values (+/- SD) of 2 independent experiments.

DISCUSSION

Chemotherapy-induced apoptosis has been considered to be induced via the mitochondrial pathway of apoptosis, in which cytochrome C release and caspase-9 activation play a central role. Some studies, however, described that chemotherapy-induced apoptosis is mediated via the death receptor pathway, in which DISC

formation and caspase-8 activation are key events. In this study, using 3 different cell lines derived from patients with acute leukemia, we investigated the role of the death receptor pathway, and in particular of caspase-8, in apoptosis induced by cytostatic agents. We observed that caspase-8 was rapidly cleaved upon exposure to camptothecin, comparable to caspase-9 and caspase-3 activation. Moreover, inhibition experiments using specific caspase-inhibitors revealed that camptothecin-induced apoptosis was at least equally effectively blocked by caspase-8 as by caspase-9 inhibitor. To examine whether this caspase-8 activation was due to activation of the death receptor pathway, we compared sensitivity of mock-transduced leukemic cell lines with FLIP-transduced cell lines. No inhibitory effect of elevated expression of FLIP on camptothecin-induced apoptosis or caspase-8 activation was observed, whereas the effective inhibition of Fas- or TRAIL-induced apoptosis in the same cell lines proved functionality of the introduced retroviral construct. In contrast, introduction of Bcl-2 resulted in almost complete inhibition of camptothecin-mediated cell death in all 3 cell lines studied, showing that chemotherapy-induced apoptosis is completely dependent on the mitochondrial pathway. Moreover, caspase-8 activation was largely inhibited indicating that caspase-8 activation is occurring downstream from the mitochondria, independent of receptor binding or DISC formation.

Whether the death receptor pathway plays a role in anticancer drug-mediated cytotoxicity had been considered controversial. Several investigators showed that anti-cancer drugs induced upregulation of Fas receptor (FasR) and Fas ligand (FasL), followed by subsequent autocrine or paracrine induction of Fas-mediated apoptosis. [12-16] Furthermore, the Fas signaling pathway could also be activated by Fas trimerization in the absence of Fas-L in cells exposed to UV irradiation [39] or various cytostatic agents. [40] Studies by Friesen *et al*. indicated that extracellular blocking of the Fas-R resulted in inhibition of doxorubicin-induced apoptosis, [12] while overexpression of FADD-DN did not provide protection against drug-induced apoptosis in Jurkat and CEM cells, [18] emphasizing the complexity of the involvement of the CD95 system on chemotherapy-induced apoptosis. The same group argued that triggering of death receptor and/or mitochondrial pathways upon drug treatment is cell type specific, proposing that in so-called type I cells both the receptor and the mitochondrial pathway are activated upon drug treatment, whereas in type II cells apoptosis is predominantly controlled by mitochondria. [18]

Here, we demonstrate that despite considerable caspase-8 activation chemotherapy-induced apoptosis of leukemic cells is not dependent on the death receptor pathway, as previously also reported by Wesselborg *et al*. for the human leukemic T-cell lines CEM and Jurkat. [26] We observed this both in an AML cell line (AML-193) and in two ALL cell lines (ALL-HP and ALL-CM) that closely resembled the primary ALL cells from which they were derived, suggesting that chemotherapy-induced apoptosis

of primary leukemic cells is also not mediated via the death receptor pathway of apoptosis.
Although recent clinical studies identified low or absent expression of FADD protein in AML cells at diagnosis as a new independent prognostic factor for poor response to chemotherapy, [20] decreased FADD expression was only associated with resistance to chemotherapy. Therefore, it is likely that another component of the death receptor pathway is responsible for the poor clinical response observed in these patients. As illustrated in our study, retroviral introduction of FLIP into AML cells seemed to be coincided with downregulation of procaspase-8 (Figure 3.6A and B). Based on the results of this study and on other studies showing that downregulation of procaspase-8 expression resulted in several malignancies in resistance to doxorubicin-induced apoptosis, [9;23-25] we suppose that defects in caspase-8 activation may underlie unresponsiveness of leukemic cells to chemotherapy treatment.

In our panel of cell lines, cleavage of caspase-8 occurred predominantly downstream of mitochondria (Figure 3.6). The mechanism of caspase-8 activation independently of death receptors and downstream of mitochondria is unknown. An early event in the induction of chemotherapy-induced apoptosis is the release of cytochrome C into the cytosol, followed by formation of the apoptosome, consisting of Apaf-1, ATP and procaspase-9, and subsequent cleavage of caspase-9 as the most apical caspase. [11;29] Caspase-8 might also bind to Apaf-1, since it has been found that caspase-8 can interact with the *Caenorhabditis elegans* cell death regulator Ced-4, a homologue of human Apaf-1, in cell free systems. [41] The long prodomain of procaspase-8 would then serve as caspase recruitment domain (CARD), which can bind to the CARD motif of Apaf-1. Procaspase-8 would compete with procaspase-9 for binding to Apaf-1, which may explain the partial inhibition of camptothecin-induced apoptosis obtained with both caspase-8 and caspase-9 inhibitor (Figure 3.4). Procaspase-8 might also be cleaved by active caspase-3 in an amplification step. [42] The results shown in Figure 3.3 do not reveal the order of caspase activation after chemotherapy-induced apoptosis, since in all 3 cell lines studied caspase-8, caspase-9 and caspase-3 activation occurred almost simultaneously. Caspase-3 activity was probably higher than caspase-8 and caspase-9 activity (Figure 3.3B) because more effector caspases (casp-3) than initiator caspases (casp-8 and -9) are required to execute apoptosis.
As expected, Bcl-2 expression did not inhibit death-receptor-mediated apoptosis (Figure 3.5B and C). Unexpectedly, we observed inhibition of death receptor-induced apoptosis by caspase-9 inhibitor (Figure 3.4), which may be explained by non-specific inhibition by irreversible caspase-inhibitors. Therefore, we suppose that in our panel of cell lines apoptosis induced by extracellularly triggering of death receptors is not dependent on the mitochondrial pathway.

In summary, understanding the mechanisms of anticancer drug-induced apoptosis is of major importance for developing effective strategies in tumor therapy.

Since caspase-8 plays a central role in both immuno- and chemotherapy-induced apoptosis of malignant cells from patients with acute leukemia, and is downregulated in certain tumors, [23-25] therapeutic strategies focusing at modulation and activation of caspase-8 may sensitize drug-resistant malignancies to radiation or combination chemotherapy. Moreover, we assume that cross resistance of CD95 resistant cells to anticancer agents which is commonly observed in leukemia patients, can be explained by defects in a common element of both pathways, such as caspase-8, instead of by activation of the death receptor pathway by cytostatic agents. To investigate this, small interfering RNA (siRNA)-mediated knockdown of caspase-8 expression or genetic ablation in mice are preferable ways of interfering with the function of caspase-8 instead of inhibition assays using caspase-inhibitors.

CONCLUSION

In conclusion, here we show that caspase-8 plays a more important role in apoptosis of leukemic cells induced by cytostatic agents than previously expected. Chemotherapy-induced apoptosis is independent of the death receptor pathway, and activation of caspase-8 occurs downstream from mitochondria, independently of death receptor triggering or DISC formation.

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